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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Group Art Unit 1652	:	PATENT APPLICATION
Examiner Kathleen M. Kerr	:	
In re application of	:	POLYNUCLEOTIDE CONSTRUCTS FOR INCREASED LYSINE
LHING-YEW LI, et al.	:	PRODUCTION
Serial No. 10/067,974	:	
Filed: February 8, 2002	:	

DECLARATION OF LHING-YEW LI PURSUANT TO 37 C.F.R. § 1.132

I, Lhing-Yew Li, declare as follows:

1. I am an inventor of the isolated polynucleotide molecule which is a subject of the above referenced U.S. patent application.

2. I am currently a Program Manager employed by Archer Daniels Midland Company.

3. I am an inventor of the subject matter of United States Patent Application No. 09/722,441.

4. My contribution as an inventor of the subject matter of United States Patent Application No. 09/722,441 included the conception, design, and construction of the gene cassettes described in that Application and set forth in its specification. I was the sole inventor of that subject matter.

I declare that the foregoing is true and correct, that all statements made on information and belief are believed to true, and, further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, imprisonment, or both,

under Section 1001 of Title 18 of the United States Code, and that any false statements may jeopardize the validity of a patent which issues from the above-identified patent application.

Date June 8, 2004

Lhing-Yew Li

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Transfer of *Brevibacterium divaricatum* DSM 20297^T, "*Brevibacterium flavum*" DSM 20411, "*Brevibacterium lactofermentum*" DSM 20412 and DSM 1412, and *Corynebacterium lilium* DSM 20137^T to *Corynebacterium glutamicum* and Their Distinction by rRNA Gene Restriction Patterns

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The results of DNA-DNA hybridization and chemotaxonomic studies indicated that the glutamic acid producers *Brevibacterium divaricatum* DSM 20297^T (T = type strain), "*Brevibacterium flavum*" DSM 20411, "*Brevibacterium lactofermentum*" DSM 1412 and DSM 20412, *Corynebacterium lilium* DSM 20137^T, and *Corynebacterium glutamicum* DSM 20300^T and DSM 20163 are members of the same species. It is proposed that all of these strains should be classified in the species *Corynebacterium glutamicum*. Another glutamic acid-producing strain, *Corynebacterium callunae* DSM 20147^T, was not related at the species level to *C. glutamicum* and should retain its separate species status. A restriction fragment length polymorphism analysis in which oligonucleotides targeted against conserved regions of 16S and 23S rRNA genes were used as hybridizing probes distinguished the individual strains. This method may be a helpful tool for strain identification.

The industrial importance of glutamic acid-producing bacteria has led to the isolation of many strains that have been classified in different genera and species. "*Micrococcus glutamicus*," which was first isolated by Kinoshita et al. (22), was later transferred to the genus *Corynebacterium* as *Corynebacterium glutamicum* (1). Subsequently, other glutamic acid producers were isolated and described; these included *Brevibacterium divaricatum* (42), "*Brevibacterium flavum*" (32), "*Brevibacterium lactofermentum*" (32), *Corynebacterium callunae* (47, 48), and *Corynebacterium lilium* (47, 48). Abe et al. (1) described three groups of coryneform bacteria that produce L-glutamic acid. The members of group I, which contains *C. glutamicum*, have been reported to have DNA base compositions of around 56 mol% G+C, and the members of groups II and III have DNA G+C contents of about 53 and 65 mol%, respectively.

The results of DNA similarity studies have indicated that *B. divaricatum*, "*B. flavum*," *C. glutamicum*, and *C. lilium* are closely related genetically (44). This close relationship is supported by similar cellular fatty acid compositions (43). However, despite the recognized similarities (1, 21, 23), these bacteria have never been combined into one species. Therefore, the taxonomy of the glutamic acid-producing coryneform bacteria is rather confusing. In particular, molecular geneticists currently working with strains that nomenclaturally belong to different genera, such as *C. glutamicum* and "*B. lactofermentum*" strains, have found almost identical primary structures for genes that encode corresponding enzymes in different strains. For example, there are only 30 differences in the published nucleotide sequences of 1,139-base-pair genomic regions that encode homoserine kinase (*thrB*) in *C. glutamicum* (33) and "*B. lactofermentum*" (29) strains, and only one of these differences would result in an exchange of an amino acid in the primary sequences of the deduced gene products.

In this report we describe a molecular taxonomic study of various glutamic acid-producing bacteria and compare these organisms with typical representatives of the genera *Corynebacterium* and *Brevibacterium*.

MATERIALS AND METHODS

Bacterial strains and cultivation. The bacterial strains which we used are listed in Table 1. Names in quotation marks are not on the Approved Lists of Bacterial Names (31, 39), have not been validly published since 1 January 1989, and therefore do not have nomenclatural standing. All organisms were grown aerobically in corynebacterium broth (10 g of casein peptone, 5 g of yeast extract, 5 g of glucose, 5 g of NaCl, 1,000 ml of water; adjusted to pH 7.2 to 7.4) at 30°C.

Physiological tests. Acid formation from carbohydrates was tested on plates containing BMCG-based medium (26) supplemented with (per 1,000 ml of medium) 50 ml of a 20% solution of a carbohydrate and 30 mg of bromocresol purple. The plates were incubated at 30°C and examined for acid production periodically for up to 2 weeks. Liquefaction of gelatin was observed in medium containing 3 g of beef extract (Difco), 5 g of peptone (Difco), and 12 g of gelatin (Merck, Darmstadt, Federal Republic of Germany) (pH 7.2). Urease production and hydrolysis of casein were detected after overnight growth on urease medium (35) and 7 days of growth on calcium-Caseinat-agar medium (Merck), respectively. Hydrolysis of starch and the results of the phosphatase reaction were determined by performing plate tests as described by Potuznik and Reissbrodt (35).

Determination of fatty acids and menaquinones. Cellular fatty acids and menaquinones were kindly determined by R. Kroppenstedt (Braunschweig, Federal Republic of Germany), using methods described previously (24).

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TABLE 1. Strains and DNA G+C contents

Species	Strain	G+C content (mol%)
C. glutamicum	DSM 20300^T	54.6
	DSM 20163	56.0
C. lilium	DSM 20137^T	52.8
C. callunae	DSM 20147^T	53.6
"*B. flavum*"	DSM 20411	54.4
"*B. lactofermentum*"	DSM 20412	54.8
	DSM 1412	54.3
B. divaricatum	DSM 20297^T	54.6
B. linens	DSM 20425^T	63.8

Isolation and purification of DNA. Bacterial cells were grown with aeration at 30°C to the late exponential growth phase in corynebacterium broth. The medium was supplemented with 0.5 to 1% glycine as recommended by Yamada and Komagata (46) in order to facilitate subsequent lysozyme lysis of the cells. At 1 h before harvesting, penicillin G was added to the cultures at a concentration of 100 U/ml. The cells were harvested by centrifugation at 4°C and washed twice with 0.15 M NaCl–0.1 M EDTA (pH 8.0). The procedure used for extraction and purification of DNA was the procedure described by Marmur (28).

G+C content of DNA. The melting point of the purified DNA was determined by thermal denaturation with a Gilford model 2600 spectrophotometer (18). DNA from *Escherichia coli* B (Sigma, Deisenhofen, Federal Republic of Germany), with a G+C content of 51.7 mol%, was used as a standard. The G+C contents were calculated by using the method of De Ley (11) and were corrected to the reference value determined for *E. coli* B DNA.

DNA-DNA hybridization studies. For DNA-DNA hybridization studies two different methods were used. In the first method we spectrophotometrically determined renaturation rates with a Gilford model 2600 spectrophotometer as previously described (12, 18). The second method was DNA-DNA dot hybridization. Chromosomal DNA from *C. glutamicum* DSM 20300^T (T = type strain) was labeled by nick translation, using [α-^{32}P]ATP (nick translation kit; BRL, Eggenstein, Federal Republic of Germany). Chromosomal DNAs (approximately 10 μg) from the corynebacterial strains were applied to a Zeta Probe nylon membrane (Bio-Rad, Munich, Federal Republic of Germany) by using a dot blot block (Schleicher & Schuell, Dassel, Federal Republic of Germany). Hybridization was performed in 0.5 M phosphate buffer (pH 7.2) containing 7% sodium dodecyl sulfate, 1% bovine serum albumin, and 1 mM EDTA at 65°C for 16 h. After hybridization the filters were washed twice at 65°C with 15 ml of 40 mM phosphate buffer (pH 7.2) containing 5% sodium dodecyl sulfate, 0.5% bovine serum albumin, and 1 mM EDTA and once at 65°C with 100 ml of 40 mM phosphate buffer (pH 7.2) containing 1% sodium dodecyl sulfate and 1 mM EDTA. After autoradiography the degrees of binding of radioactivity to the dots were determined with a video densitometer (BioTec Fischer, Reiskirchen, Federal Republic of Germany). The radioactivity bound to the DNA dots was corrected for differences in the amounts of chromosomal DNA originally loaded. To do this, the membrane-bound DNA of each dot was quantified by hybridization with a 5'-end-labeled oligonucleotide (5'-GTA GCGAAATTCCTTGTCG-3') complementary to a highly conserved 23S rDNA sequence. This method of standardization was based on the assumption that there are no great differences between the strains in the number of rRNA operons per chromosome. Oligonucleotides were labeled by using [γ-^{32}P]ATP triphosphate and T4 polynucleotide kinase (27). Hybridizations with oligonucleotides were carried out basically as described above (by using the same buffers, except that bovine serum albumin was not included) but with the following incubation periods, temperatures, and conditions: prehybridization was performed for 2 h at 65°C, the prehybridization solution was replaced with hybridization buffer, labeled oligonucleotide was added, hybridization was carried out at 40°C for 16 h, filters were washed for 10 min at 40°C and for 10 min at 45°C, and bound radioactivity was visualized by autoradiography.

RFLP analysis. For the restriction fragment length polymorphism (RFLP) analysis genomic DNA (5 to 10 μg) was cleaved with restriction endonuclease *Bam*HI, *Hin*dIII, or *Bgl*I according to the instructions of the supplier (Boehringer, Mannheim, Federal Republic of Germany). Digestions were performed at 37°C for 5 h and stopped by heating the preparations at 65°C for 10 min. DNA restriction fragments were separated by electrophoresis in 1% (wt/vol) agarose gels (Pharmacia, Freiburg, Federal Republic of Germany) with Tris acetate electrophoresis buffer as described by Maniatis et al. (27). *Hin*dIII restriction fragments of bacteriophage lambda (Boehringer) were used as size markers. After electrophoresis the agarose gels were soaked in 0.25 M HCl for 30 min and, after a brief wash with water, in 0.4 M NaOH for another 30 min. Southern transfer of DNA to Zeta Probe nylon membranes (Bio-Rad) was carried out as described by Maniatis et al. (27). The filters were air dried and subsequently baked in a vacuum oven at 80°C for 1 h. rRNA gene restriction patterns were obtained by hybridizing the preparations with an equimolar mixture of two oligonucleotides complementary to highly conserved regions of 16S and 23S rDNA sequences (the 16S rRNA-specific oligonucleotide was 5'-GTATTACCGCGGCTG-3', and the 23S rRNA-specific oligonucleotide was 5'-CCTCGATGTCGGCTC-3'). End labeling of oligonucleotides and the hybridization conditions were as described above.

RESULTS

Physiological properties. The glutamic acid-producing bacteria which we studied (*B. divaricatum* DSM 20297^T, "*B. flavum*" DSM 20411, "*B. lactofermentum*" DSM 20412 and DSM 1412, *C. callunae* DSM 20147^T, *C. glutamicum* DSM 20300^T and DSM 20163, and *C. lilium* DSM 20137^T) were very similar to each other. All produced acid from glucose, fructose, sucrose, and maltose. No acid was formed from arabinose, xylose, galactose, or mannitol. None of the organisms hydrolyzed starch, casein, or gelatin, nor did any produce phosphatase. All strains except *C. callunae* DSM 20147^T displayed urease activity. The results of more detailed investigations of physiological properties done by other workers (1, 38, 47) also reflect a high degree of metabolic similarity among these bacteria. In an extensive numerical taxonomic study of "coryneform bacteria" which included all of the glutamic acid bacteria used in this study except *C. glutamicum* DSM 20163, all of the organisms clustered in one group (38).

Chemotaxonomic properties. The G+C contents of the DNAs of the glutamic acid-producing bacteria which we investigated all fell within a very narrow range, 52.8 to 56 mol% (Table 1). These values are in good agreement with previously published data (1, 46) and lie within the range that is typical for the genus *Corynebacterium* (5). Thus, the DNA

TABLE 2. Results of DNA-DNA hybridization between *C. glutamicum* DSM 20300^T and other glutamic acid-producing corynebacteria

Strain	% Similarity to *C. glutamicum* DSM 20300^T	
	Radioactive method	Spectrophotometric method
C. glutamicum DSM 20163	73	74
C. lilium DSM 20137^T	93.3	73
"*B. flavum*" DSM 20411	87.2	87
"*B. lactofermentum*" DSM 20412	82.7	70
"*B. lactofermentum*" DSM 1412	103	74
B. divaricatum DSM 20297^T	77.9	78.8
C. callunae DSM 20147^T	36.7	37.7
B. linens DSM 20425^T	2.2	13

G+C contents of *B. divaricatum* DSM 20297^T, "*B. flavum*" DSM 20411, and "*B. lactofermentum*" DSM 20412 and DSM 1412 are significantly lower than the G+C contents reported previously for true brevibacteria (60 to 67 mol% [19]).

Previous studies have shown that all of the strains which we studied, including *Brevibacterium linens* DSM 20425^T, contain the directly cross-linked *meso*-diaminopimelic acid peptidoglycan (15, 37). Moreover, all of the strains except *B. linens* DSM 20425^T contain arabinogalactan as a cell wall polysaccharide (20, 44a). *B. linens* does not contain arabinogalactan but contains a cell wall teichoic acid (13, 14). Relatively short-chain mycolic acids (2-alkyl-branched 3-hydroxy acids), which are considered to be valuable chemotaxonomic markers for the genus *Corynebacterium* and related taxa (30), have been detected in all of the glutamic acid bacteria included in this study (7). In contrast, mycolic acids are not present in the cell envelopes of true members of the genus *Brevibacterium* (19).

The glutamic acid-producing strains which we investigated are indistinguishable on the basis of their menaquinone and fatty acid profiles (24a). They differ in menaquinone composition [predominantly MK-9(H_2)] from *B. linens* [MK-8(H_2)]. Their fatty acids are characterized by the predominant presence of saturated *n*-16 and monounsaturated 18:1 fatty acids. These data are in accordance with results reported by other workers (6, 8, 9, 44).

DNA-DNA hybridization studies. The results of the DNA-DNA hybridization studies carried out by using two different methods (see Materials and Methods) are shown in Table 2. When the radioactive dot hybridization procedure was used, high degrees of similarity were detected between *C. glutamicum* DSM 20300^T and the following strains: *B. divaricatum* DSM 20297^T, "*B. flavum*" DSM 20411, "*B. lactofermentum*" DSM 20412 and DSM 1412, *C. glutamicum* DSM 20163, and *C. lilium* DSM 20137^T (Table 2). *C. callunae* DSM 20147^T exhibited only about 37% similarity and *B. linens* DSM 20425^T shared no significant DNA-DNA similarity with the *C. glutamicum* type strain. The results of the second method showed the same trend (Table 2); *C. glutamicum* DSM 20300^T exhibited substantially less DNA-DNA similarity with *C. callunae* DSM 20147^T than with the other glutamic acid-producing bacteria and exhibited no significant similarity with *B. linens* DSM 20425^T. For some of the strains which we investigated DNA similarity values have been published previously (23, 43); these data agree reasonably well with our results.

With some strains we found significant differences in levels of similarity depending on the hybridization method used. For example, the levels of DNA-DNA similarity between *C. lilium* DSM 20137^T and *C. glutamicum* DSM 20300^T were 93.3 and 73% when we used the radioactive dot hybridization method and the spectrophotometric method, respectively (Table 2). The reasons for these discrepancies are not clear, but we assume that the quality of the DNAs (e.g., contamination by proteins, salt, or traces of organic solvents, sizes of DNA fragments) may be critical for spectrophotometric determinations because it influences the renaturation rate. On the other hand, the dot hybridization method is less demanding with respect to DNA quality. Irrespective of the methodology-associated discrepancies just described, the results of both DNA-DNA hybridization methods showed that all of the glutamic acid-producing strains which we studied except *C. callunae* DSM 20147^T exhibit at least 70% DNA-DNA similarity with the *C. glutamicum* type strain. Also, the results obtained with the two methods correspond in the low degrees of similarity between *C. glutamicum* DSM 20300^T and *C. callunae* DSM 20147^T and between *C. glutamicum* DSM 20300^T and *B. linens* DSM 20425^T.

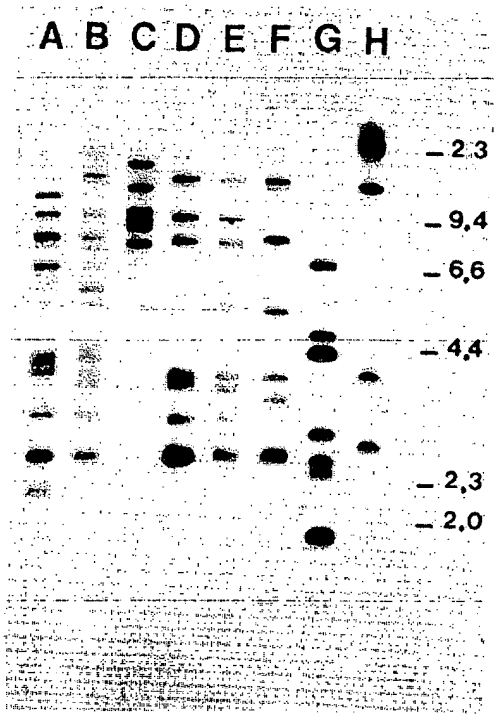

FIG. 1. rRNA gene restriction patterns for various glutamic acid-producing corynebacteria and *B. linens* DSM 20425^T. *Hin*dIII-generated genomic DNA fragments of the test strains were probed with an equimolar mixture of two oligonucleotides directed against 16S and 23S rRNA genes. For experimental details see the text. The sizes of lambda *Hin*dIII DNA molecular weight standards (in kilobases) are indicated on the right. Lane A, *C. glutamicum* DSM 20300^T; lane B, "*B. lactofermentum*" DSM 1412; lane C, "*B. lactofermentum*" DSM 20412; lane D, *C. lilium* DSM 20137^T; lane E, "*B. flavum*" DSM 20411; lane F, *B. divaricatum* DSM 20297^T; lane G, *C. callunae* DSM 20147^T; lane H, *B. linens* DSM 20425^T.

RFLP analysis. Restriction fragments of chromosomal DNAs of the test strains were hybridized with oligonucleotides directed against conserved sequences of 16S and 23S rDNAs (see Materials and Methods). We found that *B.*

TABLE 3. rDNA RFLP patterns of glutamic acid-producing strains

Fragment size (kb)	rDNA RFLP patterns								
	C. glutamicum DSM 20300^T	"*B. lacto-fermentum*" DSM 1412	"*B. lacto-fermentum*" DSM 20412	*C. lilium* DSM 20137^T	"*B. flavum*" DSM 20411	*B. divaricatum* DSM 20297^T	*C. callunae* DSM 20147^T	*B. linens* DSM 20425^T	*C. glutamicum* DSM 20163
*Hin*dIII fragments									
>23.0								+[a]	
18.0			+						
16.0		+		+	+	+			
15.0								+	
13.5			+						
12.0	+								
9.8			+						
9.4	+	+		+	+				
9.2			+						
8.9			+						
8.3						+			
8.1	+	+		+	+				
8.0			+						
6.8							+		
6.6	+	+							
5.8		+							
6.0	+								
5.4					+				
5.3		+				+			
4.8							+		
4.4							+		
4.3		+							
4.2	+	+							
4.0	+							+	
3.8	+	+		+	+	+			
3.6		+		+	+				
3.4						+			
3.1	+	+		+	+				
2.9							+		
2.8								+	
2.7	+	+		+	+	+			
2.6							+		
2.4							+		
2.2	+								
1.8							+		
*Bam*HI fragments									
>30.0							+		
23.0	+	+		+					+
22.0							+		
20.0			+	+		+			
18.0								+	
17.0	+	+							
16.0			+	+		+			
15.0							+		
13.0	+	+		+		+			+
12.0	+	+		+		+			+
11.0			+					+	
9.8	+	+		+		+			+
4.4			+						
3.6			+						
3.0			+						

[a] +, Band of genomic DNA digested with *Hin*dIII or *Bam*HI that hybridized with the oligonucleotide probes.

divaricatum DSM 20297^T, "*B. flavum*" DSM 20411, "*B. lactofermentum*" DSM 1412, *C. glutamicum* DSM 20300^T, *C. glutamicum* DSM 20163, and *C. lilium* DSM 20137^T had bands in common in the *Bam*HI digests or the *Hin*dIII digests or both (Fig. 1 and Table 3). On the other hand, the *Hin*dIII rDNA RFLP of "*B. lactofermentum*" DSM 20412 differed completely from the *Hin*dIII rDNA RFLPs of the other strains. However, when *Bam*HI-digested chromosomal DNAs were examined, this strain had bands at 16 and 20 kilobases (kb) in common with *C. lilium* DSM 20137^T and must be *B. divaricatum* DSM 20297^T (Table 3). Also, another probe consisting of a fragment of the *C. glutamicum hom-thrB* operon hybridized with a single band at an identical molecular weight from *Eco*RI-digested chromosomal DNAs of "*B. lactofermentum*" DSM 20412, *B. divaricatum* DSM 20297^T, "*B. flavum*" DSM 20411, "*B. lactofermentum*"

DSM 1412, *C. glutamicum* DSM 20300^T, and *C. lilium* DSM 20137^T (data not shown).

C. callunae DSM 20147^T did not share common rDNA bands with any of the other strains tested, irrespective of the restriction enzyme used (Fig. 1 and Table 3). Not surprisingly, *B. linens* DSM 20425^T also had RFLP patterns that were different from those of all other test strains (except an approximately 11-kb band in the *Bam*HI experiment that exhibited electrophoretic mobility similar to that of a band of "*B. lactofermentum*" DSM 20412 [Table 3]).

High degrees of banding pattern similarity were apparent for some of the strains (Fig. 1 and Table 3). For example, "*B. flavum*" DSM 20411, "*B. lactofermentum*" DSM 1412, *C. glutamicum* DSM 20300^T, and *C. lilium* DSM 20137^T all had bands at 9.4, 8.1, 3.8, 3.1, and 2.7 kb in the *Hin*dIII rDNA RFLP experiment. The "*B. flavum*" DSM 20411 pattern differed in only one band from the *C. lilium* DSM 20137^T pattern (Fig. 1, lanes D and E). In the rRNA gene restriction patterns generated with *Bam*HI, bands at 13, 12, and 9.8 kb were found with all of the glutamic acid-producing strains except "*B. lactofermentum*" DSM 20412 and *C. callunae* DSM 20147^T (Table 3). Despite these banding pattern similarities, there were enough differences to allow distinction of each strain from all of the other strains tested.

DISCUSSION

Although the morphology, staining properties, and physiological traits of *Corynebacterium* and *Brevibacterium* strains may be similar, the results of molecular studies (mainly comparative rRNA sequence studies [40, 45]) and chemotaxonomic studies have led to an improved picture of the systematic position of these genera and have simultaneously provided a basis for their distinction. The following chemotaxonomic markers are useful for distinguishing between the genera *Corynebacterium* and *Brevibacterium*: mycolic acids and arabinogalactan are present in the cell walls of *Corynebacterium* spp. (with only one exception, *Corynebacterium amycolatum*, which lacks mycolic acids [4]) but are absent in *Brevibacterium* spp., and the cell walls of *Brevibacterium* spp. contain teichoic acids which are absent in *Corynebacterium* spp. (5, 13, 19).

The results of DNA-DNA hybridization, chemotaxonomic, and biochemical studies have clearly indicated that the type strains of *B. divaricatum* (strain DSM 20297) and *C. lilium* (strain DSM 20137), as well as "*B. flavum*" DSM 20411 and "*B. lactofermentum*" DSM 20412 and DSM 1412, are so closely related to *C. glutamicum* that they should be placed in the same species. The G+C contents of all these strains are in the narrow range from 52.8 to 56 mol%. Thus, the G+C contents of the amino acid-producing brevibacteria which we studied were similar to the G+C contents of true corynebacteria but substantially lower than the G+C contents typical of true *Brevibacterium* species (60 to 67 mol% [19]). The presence of arabinogalactan as a cell wall polysaccharide and the occurrence of MK-9(H_2) and of mycolic acids, as well as the lack of cell wall teichoic acids, exclude the strains from the genus *Brevibacterium*. Analogous arguments have previously led to the transfer of two other brevibacteria to the genus *Corynebacterium*; *Brevibacterium vitarumen* and *Brevibacterium ammoniagenes* ATCC 6871^T were reclassified as *Corynebacterium vitarumen* and *Corynebacterium ammoniagenes*, respectively (3, 25). Some other strains labeled *B. ammoniagenes* (strains ATCC 13745 and ATCC 13746) have been shown to belong to *C. glutamicum* (1, 3, 23). However, the type strain of *C. ammoniagenes* is quite distinct from *C. glutamicum* (3).

The hybridization data (Table 2) indicate that *C. callunae* DSM 20147^T should have separate species status. Although distinction of this species from *C. glutamicum* on the basis of physiological and chemotaxonomic characteristics may prove to be difficult, differentiation can be achieved readily with rDNA RFLP analysis (Fig. 1 and Table 3).

rDNA restriction patterns are often visualized by using cloned rRNA genes (2, 10, 16, 36, 49), 5'-end-labeled rRNAs (17, 41), or cDNAs (34) as hybridizing probes. We used a mixture of two oligonucleotides which are targeted against conserved regions of 16S and 23S rRNA genes. The use of conserved oligonucleotides as hybridization probes has certain advantages compared with the use of the probes mentioned above. First, stringent hybridization conditions can be used since the oligonucleotides are targeted against conserved regions and therefore match perfectly. Second, oligonucleotides can be readily synthesized and labeled. Third, the intensities of the hybridization signals directly reflect the amounts of restriction fragments and are not influenced by the length of rDNA sequences present on particular fragments or by weak or unstable binding to less conserved regions of rDNA. Therefore, the pattern is not obscured by the weak bands frequently observed when rRNAs or cloned rDNAs are used as probes. In order to detect restriction sites within the 16S or 23S rRNA genes or between the 16S and 23S rRNA genes, an equimolar mixture of two probes should be used, with target sites located within the 16S rRNA gene and the 23S rRNA gene.

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